

**ATTENUATED SHIGELLA AS A DNA DELIVERY VEHICLE FOR DNA-MEDIATED IMMUNIZATION**

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**Articles main point: Use of attenuated bacteria to deliver immunizing DNA to mammalian cells.**

**ABSTRACT:**

Direct inoculation of DNA in the form of purified bacterial plasmids that are unable to replicate in mammalian cells but are able to direct cell synthesis of foreign protein is an exciting new approach to vaccine development. We have discovered that bacteria themselves are able to deliver such plasmids into the cytoplasm of cells with subsequent production of functional foreign protein. We constructed a highly attenuated bacterial vector which invades mammalian cells, breaks out of the phagocytic vacuole and ruptures delivering functional plasmid DNA into the cell cytoplasm. This *Shigella* vector was designed to deliver DNA to colonic mucosa, thus opening the possibility of oral and other mucosal DNA immunization and gene therapy strategies.

Direct DNA-mediated immunization is an exciting new approach to vaccine development. Highly purified bacterial plasmid DNAs expressing desired proteins under the control of viral promoters have been injected primarily into muscle or skin by traditional needle and syringe or by other more exotic methods such as biostatic transfection with DNA-coated gold microparticles (for review see ref. 1). Investigators using this technology have been able to elicit neutralizing antibodies, cytotoxic T lymphocytes and protection to challenge in several animal models of infection ranging from influenza to malaria (for review see ref. 1). We now report on the use of attenuated bacteria as a unique method of delivering DNA to mammalian cells. This finding has the potential to provide a simple, inexpensive way of extending DNA immunization to the local immune system and beyond through oral and other mucosal routes of immunization.

*Shigella* strains invade colonic epithelial cells of man. We choose to exploit the ability of *Shigellae* to enter epithelial cells and escape the phagocytic vacuole as a method to direct plasmid DNA to the cytoplasm of the host cell for protein synthesis and processing for antigen presentation (2). A mutation in the gene encoding aspartate  $\beta$ -semialdehyde dehydrogenase (ASD) was placed in *Shigella flexneri* 2a strain 2457T for the specific purpose of delivering DNA to mucosal epithelial cells of the gut. We present data on this strain's ability to successfully deliver a eukaryotic expression vector encoding the *E. coli*  $\beta$ -galactosidase reporter gene to mammalian cells in culture. Immunostaining and biochemical studies detected  $\beta$ -galactosidase activity at 24 and 48 hours after bacterial entry at a time when bacterial viability inside cells had markedly decreased.

A *Shigella* DNA carrier, which was sufficiently attenuated and in essence programmed for death after leaving the phagocytic vacuole, was required. In constructing an appropriate strain, we choose to take advantage of the already popular

conditional-lethal mutation system. A deletion mutation was made in the gene encoding ASD, an essential enzyme required for synthesizing the bacterial cell wall constituent diaminopimelic acid (DAP) (3). Figure 1 illustrates the construction of 15D, a  $\Delta$ asd isolate of *Shigella flexneri* 2a strain 2457T. Strain 15D was able to maintain the commercially available eukaryotic expression vector pCMV $\beta$  without antibiotic selection. pCMV $\beta$  expresses *E.coli*  $\beta$ -galactosidase under the control of the immediate early promoter and enhancer from the human cytomegalovirus (CMV) in mammalian cells, which permitted us to easily analyze mammalian-mediated gene expression after delivery (4).

Strain 15D was screened to ensure that the large plasmid essential for bacterial invasion of mammalian cells had not been lost during the genetic manipulations. Strain 15D was found to express IpaB and IpaC polypeptides as determined by immunoblotting (5) showing no loss of the invasion plasmid. It was important to demonstrate that *Shigellae* containing a mutation in a gene required for cell wall synthesis could still adhere to and invade cells in culture. Strains 15D and 15D(pCMV $\beta$ ) were each tested for the ability to invade cultured baby hamster kidney (BHK) cells with and without supplementation of DAP during the 90 minutes allowed for invasion (6). After this period of interaction, monolayers were extensively washed and treated with gentamicin containing medium for at least 30 minutes to eliminate extracellular bacteria. Both constructs were found to invade BHK cells; however, the addition of DAP during bacterial-cell interaction significantly increased the number of 15D and 15D(pCMV $\beta$ ) colonies recovered (Table 1). Fixed and stained chamberslides of infected BHK cell monolayers examined by light microscope verified viability findings. Without the presence of DAP during the invasion step, 15D and 15D(pCMV $\beta$ ) entered just 13% and 10% of the BHK cells, respectively. By contrast, 33% (15D) and 29% [15D(pCMV $\beta$ )] of the BHK cells contained bacteria when DAP was included. Since the purpose of this study was to determine if bacteria could be used to deliver plasmid DNA to mammalian

cells, DAP was added to concentrated bacteria during the adherence and invasion step in the following representative data.

Intracellular bacterial viability and  $\beta$ -galactosidase activity were followed over a 48 hour time course (Figure 2a and b) (7). Viable bacterial counts were determined by plating a 0.2% Triton-X-100 lysis sample of the monolayer.  $\beta$ -galactosidase activity was measured in the remaining cell extract by a standard biochemical assay that uses the conversion of  $\alpha$ -nitrophenol- $\beta$ -D-galactoside (ONPG) to galactose and the chromophore  $\alpha$ -nitrophenol to quantitatively detect activity spectrophotometrically. Initially  $1-3 \times 10^7$  viable bacteria of each strain were recovered from monolayers of BHK cells with no detectable  $\beta$ -galactosidase activity in cell extracts. Measurements of  $\beta$ -galactosidase activity in bacterial extracts equivalent to the total number of bacteria added were negative. After 4 hours, a log to a log and half loss in viable bacteria occurred with no detectable  $\beta$ -galactosidase activity. An additional log to log and half loss of viable bacteria was observed at both the 24 and 48 hour assay points. At both times, increasing units of  $\beta$ -galactosidase activity were readily detectable in cell extracts from BHK cells infected with 15D(pCMV $\beta$ ).  $\beta$ -galactosidase activity detected at these last assay points was not due to expression from within the bacteria because no activity was detected at the first two assay points, yet a high level of viable bacteria were present. In addition, a noninvasive isolate of 15D(pCMV $\beta$ ) (i.e., IpaB and IpaC immunoblot negative) was tested for the ability to deliver plasmid DNA. No  $\beta$ -galactosidase activity was detected at the 24 hour assay point. This finding reinforces the hypothesis that to deliver DNA the bacteria must be capable of entering the mammalian cell and breaking out of the phagocytic vacuole, which most likely occurs during the first 4 hours of this assay. By the 24 and 48 hour assay points, sufficient time had passed for lysis of the bacterium and release of the plasmid DNA into the cell cytoplasm. This is followed by transcription and translation of the encoded reporter gene. Extracellular lysis of bacteria leading to the release of plasmid DNA with

subsequent uptake by eukaryotic cell cannot account for these findings since the noninvasive isolate was unable to induce  $\beta$ -galactosidase activity.

To verify the delivery of pCMV $\beta$  DNA to BHK cells, infected monolayers were immunostained to visually detect intracellular  $\beta$ -galactosidase expression within individual cells (Figure 3) (8). No apparent intracellular immunostaining was observed in monolayers infected with either strain at the 30 minute assay point (Figure 3A,B). Only slight intracellular immunostaining was detected at the 4 hour assay point in monolayers infected with 15D(pCMV $\beta$ ) (Figure 3C,D). At the 24 and 48 hour assay points, several cells per field of monolayers infected with 15D(pCMV $\beta$ ) were positively stained (Figure 3E,F). Staining throughout the cell cytoplasm indicated that the plasmid DNA had been released from the bacterium into the cell cytoplasm for further processing (i.e., transcription and translation) by the mammalian cell. Positively staining cells also appeared to be rounded, possibly due to the presence of an extensive amount of  $\beta$ -galactosidase protein. Approximately 1-2% of 5000 cells were stained positive for  $\beta$ -galactosidase expression at the 24 hour assay point as determined by fluorescence activated cell sorter (FACS) analysis (7). Visual examination of Leukostat stained chamberslides of 15D(pCMV $\beta$ ) infected BHK cells demonstrated that 28% of the cells contained 1 to 5 intact bacterial cells with 1.7% containing 5 bacteria (Table 2). Four hours after gentamicin treatment 26% of the cells contained visually intact bacteria with less than 1% of the cells containing 4 bacteria. Therefore, invasion with between 1-5 bacteria was required for foreign gene expression. Since pCMV $\beta$  is a 7164 base pair plasmid of medium to high copy number with approximately 500 copies per bacterial cell, each bacterium is estimated to contain about 3.93 ( $10^{-9}$ )  $\mu$ g of DNA. Intracytoplasmic delivery of approximately  $4-20 \times 10^{-9}$   $\mu$ g of DNA by *Shigella* is sufficient for expression of  $\beta$ -galactosidase.

*Shigella* species invade many different types of cells. To demonstrate that gene delivery was not restricted to BHK cells, P815 cells were infected with 15D(pCMV $\beta$ ). As

shown in Table 3, 10 fold higher levels of  $\beta$ -galactosidase were expressed compared to background control at 24 hours. P815 cells, which express H-2<sup>d</sup> class I MHC molecules, have been successfully infected with 15D(pCMV $\beta$ ) and experiments are currently underway to determine if these cells can present *Shigella* delivered DNA encoded foreign antigens in the context of class I.

We have discovered a novel method for delivering functional DNA inside cells. This method should not be restricted to *Shigella*, since the invasion genes that *Shigella* utilizes can be inserted into other bacteria such as *E. coli* (9). Likewise, other bacteria such as *Listeria* are able to invade cells and break out of the phagocytic vacuole into the cytoplasm (10). Although we have no formal proof that release from the phagocytic vacuole into the cell cytoplasm by the bacteria is essential for DNA delivery, preliminary experiments with *Salmonella typhimurium*, an organism that reaches the cytoplasm only with difficulty, suggests this organism is not an efficient DNA delivery vehicle.

Any bacterial vector DNA delivery system will need to strike a balance between cell invasion with its subsequent reactogenicity and efficiency of delivery. In the case of *Shigella*, the genes responsible for invasion also cause invasion and apoptosis of macrophages followed by inflammation (11). We constructed a *Shigella* strain that in the absence of DAP is completely unable to divide. Determination of the safety of this strain awaits human trials. Preliminary experiments in a guinea pig keratoconjunctivitis challenge model demonstrate 100% protection from subsequent *Shigella* infection three weeks following a two dose immunization regime (12). This demonstrates that this highly attenuated strain, which is capable of DNA delivery, functions well *in vivo*.

The bacterial DNA delivery system which we describe has several advantages for certain applications. Delivery of DNA encoded antigens to the mucosal immune system should permit mucosal immunization simultaneously with multiple antigens that can be directed for class I and/or II presentation, stimulation of Th1 or Th2 help, or secreted maintaining the proper folding and conformational epitopes for IgA and IgG

antibody production. Diarrheal diseases such as rotavirus; sexually transmitted diseases such as human immunodeficiency virus, *Neisseria gonorrhoeae*, and human papilloma virus; and gastrointestinal diseases such as the ulcer causing *Helicobacter pylori* may be especially responsive to this approach. Suppression of autoimmunity through manipulation of gut immune tolerance mechanisms has been demonstrated (13), and if generally applicable should also be amenable to this approach.

Perhaps the greatest advantage of bacterial delivery of DNA for vaccination and potential gene therapy/replacement is the ease and acceptability of oral and other forms of mucosal delivery. Likewise, because no DNA purification is required for this type of DNA vaccination, which is really a live, attenuated bacterial vector, vaccines can be produced for the cost of fermentation, lyophilization and packaging. Therefore, this type of vaccination may represent at least in part a solution to the cost and difficulty of current vaccines and those that are being developed.

Aside from the practical application of bacterial DNA delivery, our demonstration of the relatively efficient ability of *Shigella* to transfer functional DNA containing a eukaryotic promoter to mammalian cells leads to speculation concerning the potential role of such a mechanism in evolution. Plasmids for vaccine use are designed in a manner to minimize the possibility of chromosomal integration, but of course in nature this may not be the case.

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